PB86-175486

# Development of Methods to Measure Virus Inactivation in Fresh Waters

James N. Gamble Inst. of Medical Research
Cincinnati, OH

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

1985

U.S. Department of Commerce
National Technical Information Service
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| 1. REPORT NO. | 2. | 3. REC |
|---|---|---|
| EPA/600/J-85/353 | | PB86-175486 |

| 4. TITLE AND SUBTITLE | 5. REPORT DATE |
|---|---|
| Development of Methods to Measure Virus Inactivation in Fresh Waters (Journal Version) | 1985 |
| | 6. PERFORMING ORGANIZATION CODE |
| 7. AUTHOR(S) | 8. PERFORMING ORGANIZATION REPORT NO. |
| Richard L. Ward & Pat E. Winston | |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS | 10. PROGRAM ELEMENT NO. |
| | CAYB1B |
| James N. Gamble Institute of Medical Research<br>2141 Auburn Avenue<br>Cincinnati, Ohio 45219 | 11. CONTRACT/GRANT NO.<br>CR-811183-01-0 |
| 12. SPONSORING AGENCY NAME AND ADDRESS | 13. TYPE OF REPORT AND PERIOD COVERED |
| Health Effects Research Laboratory<br>Office of Research and Development<br>U.S. Environmental Protection Agency<br>Research Triangle Park, NC 27711 | Journal Article - 10/1/83-2/28/85<br>14. SPONSORING AGENCY CODE<br>EPA-600/11 |

15. SUPPLEMENTARY NOTES

P.O. D. A. Brashear, MB/TMD/HERL-Ci

REF: Applied and Environmental Microbiology, 50(5):1144-1148, November 1985

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field/Group |
|---|---|---|
| | | |

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SPRINGFIELD, VA. 22161

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS *(This Report)* | 21. NO. OF PAGES |
|---|---|---|
| Release to public | unclassified | 6 |
| | 20. SECURITY CLASS *(This page)* | 22. PRICE |
| | unclassified | |

EPA/600/J-85/353
JOURNAL ARTICLE

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1985, p. 1144–1148
0099-2240/85/111144-05$02.00/0
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Vol. 50, No. 5

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# Development of Methods to Measure Virus Inactivation in Fresh Waters

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Received 4 March 1985/Accepted 10 August 1985

**This study concerns the identification and correction of deficiencies in methods used to measure inactivation rates of enteric viruses seeded into environmental waters. It was found that viable microorganisms in an environmental water sample increased greatly after addition of small amounts of nutrients normally present in the unpurified seed virus preparation. This burst of microbial growth was not observed after seeding the water with purified virus. The use of radioactively labeled poliovirus revealed that high percentages of virus particles, sometimes >99%, were lost through adherence to containers, especially in less turbid waters. This effect was partially overcome by the use of polypropylene containers and by the absence of movement during incubation. Adherence to containers clearly demonstrated the need for labeled viruses to monitor losses in this type of study. Loss of viral infectivity in samples found to occur during freezing was avoided by addition of broth. Finally, microbial contamination of the cell cultures during infectivity assays was overcome by the use of gentamicin and increased concentrations of penicillin, streptomycin, and amphotericin B.**

Despite recent improvements in sewage treatment practices, surface waters in developed as well as developing countries still receive high concentrations of pathogenic microorganisms. These waters are eventually used by humans, and often this use involves consumption or other direct contact. In cases where no treatment occurs before human contact, the only environmental barriers preventing the spread of disease through water are dilution, natural inactivation, and removal of enteric pathogens by processes such as adsorption to particulates and subsequent sedimentation.

Numerous studies have been conducted on the survival of pathogenic microorganisms in fresh and marine waters. Studies performed with enteric viruses indicate that loss of infectivity occurs at a variety of rates. In addition, there may be little consistency in inactivation rates among different water samples (1). The interpretation and applicability of these results, however, have been limited by deficiences in methodology.

One major flaw has been the inability to account for loss of infectious viruses due to adhesion to sample containers. Because of this deficiency, it is impossible to state with certainty that previously reported decreases in recoverable infectious viruses were due to true inactivation. Possible exceptions were studies reported by O'Brien and Newman (4) and Toranzo et al. (6). These investigators showed that radioactively labeled enteroviruses released their RNA molecules when incubated in different water samples. In the former case, the RNA molecules were also found degraded in the river waters being tested. Even these investigators, however, did not determine the fraction of lost infectivity due to adhesion rather than true inactivation. It should be noted that Akin et al. (2) recognized the possible severity of this problem and attempted to elute potentially adhering viruses from the sides of their containers.

While developing methods to conduct virus inactivation experiments, other potential deficiencies in routinely used techniques were recognized. These deficiencies may have had dramatic effects on previously reported inactivation results and subsequent interpretations. The main objective of this paper is to identify any major deficiency associated with methods used to measure virus inactivation rates. Once recognized, these deficiencies were corrected by replacement with better techniques.

## MATERIALS AND METHODS

**Virus and cells.** Attenuated poliovirus-1 strain CHAT obtained from the American Type Culture Collection (ATCC; Rockville, Md.) was grown in HeLa cells (our stock) and assayed for infectivity by plaquing on human rhabdomyosarcoma cells, also obtained from ATCC. HeLa cells were grown in Eagle minimal essential medium (MEM), and rhabdomyosarcoma cells were grown in special MEM (Richter modification; Irvine Scientific Co., Santa Ana, Calif.). Both media were supplemented with 10% fetal calf serum and antibiotics (100 U of penicillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B per ml). Plaque assays were performed as previously described (7) with an overlay medium consisting of special MEM with 5% fetal calf serum, antibiotics, and 0.18% agarose.

**Labeling and purification of poliovirus.** Confluent monolayers of HeLa cells in 150-$cm^2$ flasks were infected with poliovirus-1 (multiplicity of infection, 10 to 50). After a 30-min adsorption period, 20 ml of MEM with 5% fetal calf serum was added to each flask, and incubation at 37°C was continued for an additional 2 h. [$^3$H]uridine (0.5 mCi) was then added, and incubation was continued for 16 to 20 h until cytopathic effects were complete. Viral lysates were frozen at −20°C, thawed, and blended with an equal volume of cold Freon. After centrifugation (2,000 × *g*, 20 min) to separate phases, the upper aqueous phase was collected and layered over 8 ml of CsCl (1.4 g/ml) in an SW27 centrifuge tube. The virus was banded in the CsCl cushion (24,000 rpm, 4 h) and collected by syringe through the side of the tube. The density of the collected sample was adjusted to 1.34 g/ml, and the virus was further purified by isopycnic gradient centrifugation (24 h, 30,000 rpm, SW50.1 rotor). The virus band that formed during centrifugation was collected, dialyzed against NTE buffer (0.1 M NaCl, 0.01 M Tris [pH 7.5], 0.001 M EDTA), and stored at −70°C.

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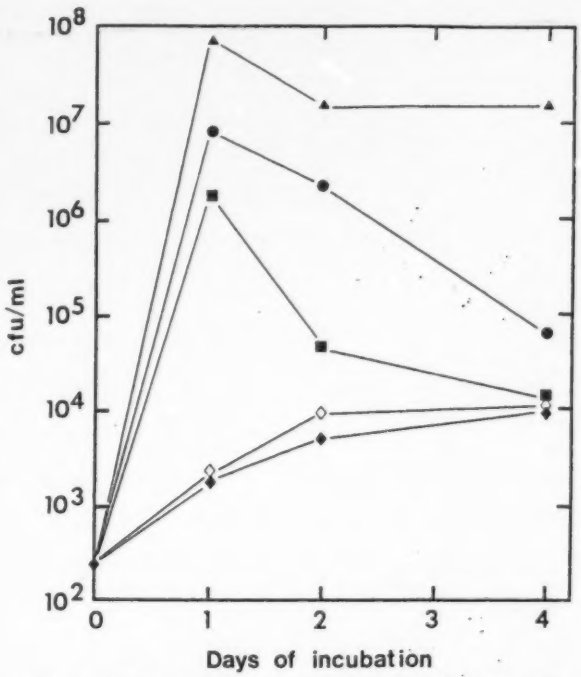


FIG. 1. Effect of nutrients in tissue culture media on the growth of TPCs of organisms in creek water. Samples of creek water were inoculated with a 10-fold (▲), 100-fold (●), or 1,000-fold (■) dilution of tissue culture media used to support HeLa cells during growth of poliovirus and were incubated on a rotary shaker (180 rpm) at 26°C. Samples were taken on the day specified, and TPC was determined. The same water was either left unseeded (◆) or seeded with purified poliovirus (◇) before incubation and sampled at the same time periods. Results are presented as CFU per milliliter.

**Analysis of water samples.** In this study, most environmental water samples used were collected from streams with various degrees of pollutants that were located within a 25-mile radius of Cincinnati. Other water sources included a deep well, an unpolluted lake, the Ohio River, and a commercial spring water. All waters were stored at 4°C after collection and were analyzed for total plate count (TPC) of organisms, turbidity, pH, and conductivity within 24 h. TPC was determined by mixing 0.1 or 1.0 ml of serially diluted water samples with 15 ml of nutrient agar 45°C in 100-mm (diameter) petri dishes. After the agar solidified, the plates were incubated at 26°C for 48 h, and the colonies were counted. Turbidity, pH, and conductivity were determined with an HF model DRT 15 turbidimeter, a Corning digital 110 pH meter, and a YSI model 31 conductivity bridge, respectively.

## RESULTS

**Properties of virus preparations needed to conduct inactivation studies in fresh waters.** The effects of different fresh waters on the survival of enteric viruses can be determined by seeding these waters with a known concentration of virus and measuring loss of infectivity. Viruses used in these studies are grown in cells maintained in tissue culture media. These media contain nutrients that should also support bacterial growth. Since microorganisms in environmental waters are typically starved for nutrients, the addition of even small amounts of tissue culture media to these waters could potentially cause large increases in microbial concentrations. In turn, natural inactivation rates of viruses in these waters could be altered. Therefore, the first experiments were to determine whether unpurified viruses in tissue culture media could be used to seed water samples without affecting microbial concentrations.

The tissue culture medium used to support cells during growth of poliovirus-1 was diluted 10-, 100-, and 1,000-fold into creek water initially containing $2.5 \times 10^2$ TPC of organisms per ml. After 1, 2, and 4 days of incubation at 26°C, the TPCs in these cultures were compared with those in the same water incubated without nutrients. Dramatic increases in TPCs were observed during the first 24 h in all samples supplemented with nutrients, even when nutrients were diluted 1,000-fold (Fig. 1). TPCs declined in these samples after day 1. A much smaller but gradual increase in TPC was observed in the samples incubated without added nutrients. Shuval et al. (5) made a similar observation when seawater was seeded with a 1,000-fold dilution of tissue culture fluid.

Since greater dilutions of the viral lysate could limit the usefulness of the results and may still promote microbial growth, extensive reduction or elimination of nutrients was needed in the virus preparation. This was accomplished by a series of virus purification steps described in Materials and Methods. Viruses purified in this manner and seeded (1,000-fold dilution) into creek water had little effect on the gradual increase in TPC observed in the unseeded water sample during 4 days of incubation (Fig. 1).

One of the greatest potential causes for inaccuracy in measuring virus inactivation rates in water samples is loss due to adherence to the container. Corrections for such losses could be made if the viruses were radioactively labeled. Recoverable viral infectivity would then be measured as specific infectivity or PFU per counts per minute.

To determine whether radioactive labeling was required to account for losses due to adherence, purified [$^3$H]uridine-labeled poliovirus-1 was mixed with water samples from different sources. Loss of recoverable radioactivity was then measured as a function of incubation time (26°C) in stationary Pyrex glass (borosilicate) containers. Results obtained with five representative water samples are shown in Fig. 2. Virus loss due to adherence occurred immediately upon mixing the virus with water. This loss was greater in some waters than in others. Larger differences in total virus losses among waters were seen after 1 day of incubation, but some further reduction in recoverable radioactivity was found in every water tested. A portion of the radioactivity lost during the first several days, however, was recoverable in all waters tested after longer periods of incubation.

Examination of water properties relative to loss of virus by adherence revealed that the less turbid environmental waters caused the greatest losses. A direct relationship was found between turbidity (expressed in nephelometric turbidity units [NTU]) and radioactivity recovered after 1 day of incubation with [$^3$H]uridine-labeled poliovirus (Fig. 3). This relationship was not observed when poliovirus was seeded into laboratory-distilled water in Pyrex glass from which high virus recoveries were invariably obtained (data not shown).

**Optimization of methods to avoid virus adherence to containers.** Experimental conditions were reevaluated to reduce the large virus losses due to adherence to containers. Material composition of the containers was the first condition to be tested. Certain plastics are hydrophobic and may be more suitable than glass for this type of experimentation. There-

fore, adherence of [$^3$H]uridine-labeled poliovirus-1 was measured in test tubes composed of Pyrex glass, polystyrene, and polypropylene. The water samples selected were two that had caused the greatest adherence in the previous study, a filtered creek water and a commercial spring water.

Some adherence of labeled poliovirus occurred within the first few minutes in all containers tested in both creek water (Fig. 4A) and spring water (Fig. 4B). Pyrex glass caused the most rapid adherence, and polypropylene (the most hydrophobic material) appeared to be the least adsorptive material. Even with polypropylene, however, nearly 90% of the radioactivity adhered to the walls of the container in the spring water. Because polypropylene was the best of the materials tested, containers of this material were used in all subsequent experiments. It should be noted that siliconization of the glass containers did not reduce virus adsorption (data not shown).

Movement (shaking, rotation, or rolling) of sample containers during incubation could also affect adherence of virus. Therefore, experiments were conducted to compare binding of radioactively labeled poliovirus-1 in rotated (180 rpm) versus stationary flasks, using 10 different water samples. Because the result of the previous experiment was not yet available, this particular study was conducted with glass flasks. Losses were greater in the rotated flasks in all 10 waters. The average recoverable radioactivity for these waters under the two incubation conditions is shown in Fig. 5. In all subsequent experiments, flasks were held stationary during incubation and were shaken only before they were sampled to insure proper mixing.

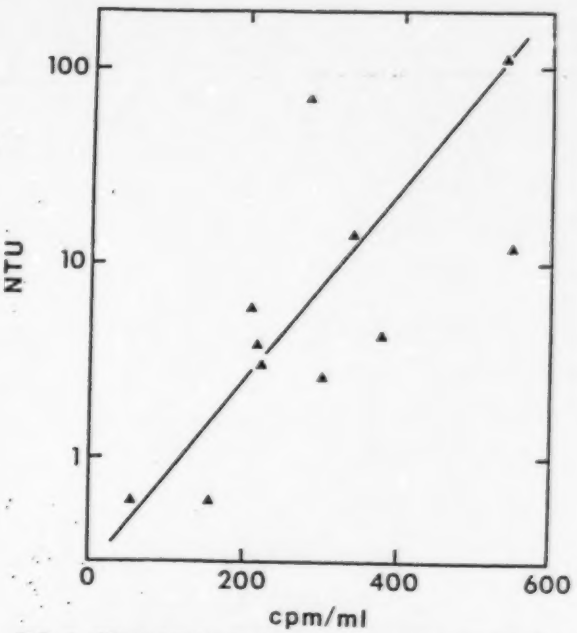


FIG. 3. Effect of turbidity (NTU) of different waters on adhesion of radioactively labeled polioviruses to container walls. An average of 1,027 cpm of [$^3$H]uridine-labeled poliovirus per ml was seeded into different waters, and the flasks (Pyrex glass) were held stationary during incubation. Recoverable radioactivity shown in this plot was determined after 1 day at 26°C.

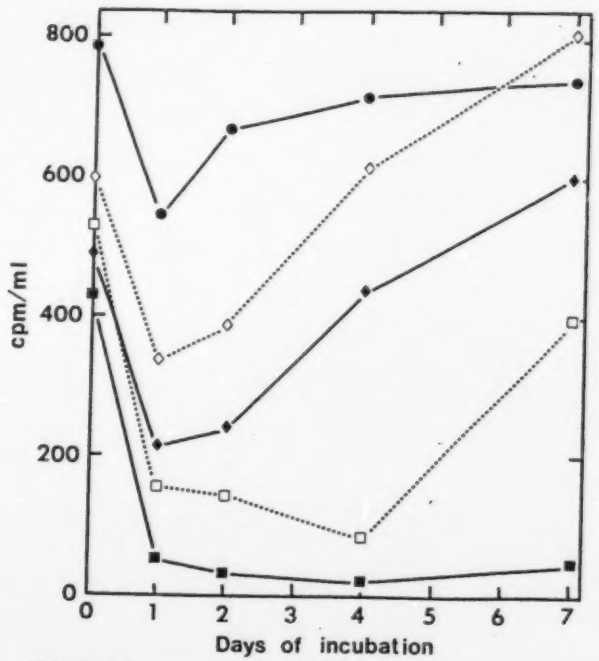


FIG. 2. Adherence of radioactively labeled poliovirus to the walls of containers after addition to different waters. [$^3$H]uridine-labeled poliovirus was diluted 1,000-fold into different waters (20 ml) in Pyrex glass flasks (125 ml) and held stationary during incubation at 26°C. Portions (2 ml) were sampled at specified intervals and analyzed in duplicate for recoverable radioactivity. An average of 1,027 cpm/ml was added to each of the flasks. The waters shown included a well water (●), a polluted stream (◇), an unpolluted stream (◆), a filtered (0.2 μm) unpolluted stream (□), and a commerical spring water (■).

**Optimization of sample storage conditions.** When water samples seeded with a virus were collected, they were always frozen and stored at −20°C until they were assayed

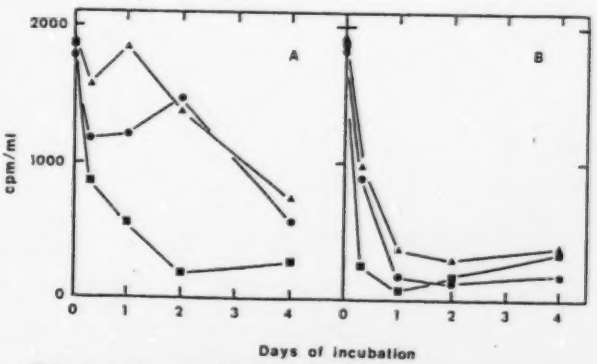


FIG. 4. Adherence of [$^3$H]uridine-labeled poliovirus to Pyrex glass (■), polystyrene (●), and polypropylene (▲) when seeded into filtered creek water (A) or commercial spring water (B). An average of 2,255 cpm of labeled virus per ml was recoverable from each water before distribution of 3 ml into duplicate test tubes composed of the different materials. The tubes were rolled during incubation (26°C), and samples (0.5 ml) were counted at the specified intervals. The first sample was collected within 5 min from the time the seeded waters were added to the test tubes.

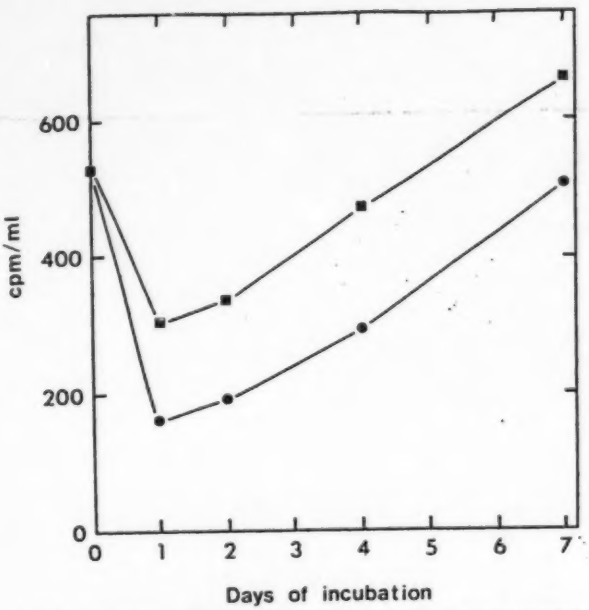


FIG. 5. Average recoveries of radioactivity from waters seeded with [$^3$H]uridine-labeled poliovirus and incubated either stationary (■) or with rotation (●). Water samples from 10 different sources were seeded with an average of 1,027 cpm of labeled poliovirus per ml, and 20 ml was added to separate Pyrex glass flasks (125 ml). The flasks were either rotated (180 rpm) or left stationary at 26°C. Portions were taken at the specified times and analyzed for recoverable radioactivity.

for recoverable radioactivity and infectivity. In water samples collected immediately after seeding with an equal volume of poliovirus-1, specific infectivities of the virus often varied by more than 1 order of magnitude. This finding indicated that a variable amount of virus inactivation occurred between the time of collection and the analysis of samples. It was also observed that samples could not be refrozen without considerable reduction in specific infectivity, sometimes greater than 99%. These results indicated that freezing the water samples without the addition of protective components could cause extensive virus inactivation.

Loss of virus infectivity during freezing was avoided by dilution of the water samples into tryptose-yeast extract (TYE) broth (4 volumes of water plus 1 volume of 5× TYE broth). This change allowed full protection during freezing when tested with poliovirus-1 in a creek water (data not shown). In subsequent experiments, it was found that the inconsistencies in specific infectivities observed in samples collected immediately after seeding with poliovirus-1 were greatly reduced when these samples were frozen in TYE broth. Initial specific infectivity values for any set of samples were consistently less than two-fold different from the mean value. Also, no reduction in titer was observed in samples that were refrozen. Thus, addition of TYE broth appeared to be an effective method of retaining the infectivities of enteric viruses in frozen water samples.

**Reduction of microbial contaminants during plaque assay.** Although environmental waters have few nutrients to support microbial growth, the waters collected for this study still typically contained a TPC of $10^2$ to $10^4$ organisms per ml. The numbers of these organisms are sufficiently high to contaminate the cell cultures during plaque assay and obscure the results. "Normal" concentrations of standard antibiotics and dilution of the samples could reduce or eliminate this contamination. Contamination was frequently observed, however, in tissue culture plates in samples receiving little or no dilution. This was particularly true in samples with higher TPCs.

A simple and potentially effective method for reducing microbial contamination of tissue culture media during plaque assay is to use additional antibiotics. This method was tested with several concentrations of penicillin, streptomycin, amphotericin B, and gentamicin. These antibiotics were added to the agarose overlay medium after inoculation of monolayer cultures of rhabdomyosarcoma cells with different dilutions of activated sludge obtained from a local sewage treatment plant. Activated sludge was used because it had a consistently higher TPC than the enviromental waters tested. Also, the microorganisms in activated sludge were potentially protected by particulates, thus making this a "worst case" situation.

Both the absence of antibiotics and the use of standard concentrations of normal antibiotics (i.e., 100 U of pencillin, 100 μg of streptomycin, and 2.5 μg of amphotericin B per ml) in the plaque assay overlay medium resulted in total destruction of the cell monolayer within 2 days of incubation because of microbial growth (Table 1). This destruction occurred even when activated sludge was diluted 2 orders of magnitude before addition to the cells. When higher concentrations of normal antibiotics were used in conjunction with gentamicin, cell destruction by microbial contamination was reduced considerably. An eightfold increase in normal antibiotic concentrations plus gentamicin (200 μg/ml) completely blocked visible microbial growth in the undiluted sample after a 2-day incubation period. However, this concentration of antibiotics also caused slight cell toxicity. Therefore, 2× normal antibiotics with 50 μg of gentamicin per ml were incorporated into the overlay medium for subsequent experiments. This concentration caused no apparent reduction in plaque numbers. Also, no visible microbial growth occurred during the plaque assay with any other water sample, even when tested undiluted.

TABLE 1. Effect of different antibiotic concentrations on microbial growth in plaque assay overlay medium[a]

| Antibiotic | Microbial growth[b] | | | |
|---|---|---|---|---|
| | 2 days | | 3 days | |
| | Undiluted | 100-fold diluted | Undiluted | 100-fold diluted |
| None | 3+ | 3+ | 3+ | 3+ |
| Normal | 3+ | 2–3+ | 3+ | 2–3+ |
| 2× Normal[c] + 50 μg of gentamicin per ml | 3+ | 0 | 3+ | 0 |
| 4× Normal + 100 μg of gentamicin per ml | 3+ | 0 | 3+ | 0 |
| 8× Normal + 200 μg of gentamicin per ml | 0 | 0 | 1+ | 0 |

[a] Activated sludge (0.2 ml), either undiluted or diluted 100-fold in balanced salt solution, was adsorbed onto confluent monolayers of rhabdomyosarcoma cells (1 h, 37°C). Overlay medium (special MEM, 5% fetal calf serum, 0.18% agarose) with different concentrations of antibiotics was added (5 ml), and plates were incubated for either 2 or 3 days before being observed for microbial contaminants. Plates were also stained with crystal violet after removal of the soft agarose overlay to determine the effect of antibiotics on cell monolayers. The experiment was performed in duplicate.

[b] Scale of microbial growth: 0, no visible growth; 1+, slight growth; 2+, moderate growth; 3+, confluent growth.

[c] Normal antibiotic concentrations are specified in text.

## DISCUSSION

Most studies concerned with virus behavior in water, soils, and other environmental samples have relied solely upon one assay to detect the presence of infectious viruses. This is the plaque assay. In some instances it was assumed that loss of viral PFUs meant that the virus particles had been physically removed; in other cases it was assumed that they had been inactivated. Little or no evidence was presented to support either conclusion, and yet numerous examples of each are found in the literature.

This report shows that sole reliance on the plaque assay can lead to serious misinterpretation of the results. Viruses seeded into water samples adhered to the sides of the containers. The degree of adherence varied among the different waters and was shown to be related to the turbidities of the waters. In some cases, more than 99% of virus particles became bound within 1 to 2 days of incubation, even in flasks held stationary during incubation. This was probably because of the scarcity of competitive binding substances in these waters. Loss of virus due to adherence was measurable because the viruses were radioactively labeled, a method that could be used to standardize results in many environmental studies.

Adherence of viruses to the walls of containers was not an irreversible process. In every water sample tested, a portion of the radioactivity that was lost during the first days of incubation was released within 7 days. Since the nucleic acid was the radioactive component of the virus in these studies, recovery of radioactivity from the walls of containers could have been due to breakdown of adhering particles and subsequent release of viral RNA. A subsequent study has revealed that loss of poliovirus infectivity occurred in proportion to the release of RNA from poliovirus particles in one of these waters (P. E. Winston and R. L. Ward, submitted for publication).

To use radioactively labeled viruses in these experiments, the viruses must also be purified to remove extraneous radioactive components. The presence of such components could cause misinterpretation of the results if their adherence properties were different from those of the viral particles. Purification of the viruses was also shown to remove nutrients that, if added to the waters, could cause microbial growth. This, in turn, could alter the survival characteristics of the viruses. Very small amounts of nutrients contained in unpurified virus preparations were shown to cause large increases in viable microorganism concentrations. Addition of purified viruses to the water samples did not have this effect.

Other methods were developed to standardize this type of experimentation. Most of these methods are applicable to other types of investigations in environmental virology. The use of polypropylene sample containers held stationary during incubation allowed considerable reduction in virus losses due to adherence, an observation also made by other investigators who used labeled poliovirus in a buffered salt solution (3). This was especially true for relatively clean waters. If stored frozen before being assayed, water samples containing a virus needed a protective component. The use of TYE broth was effective for this purpose, not only for poliovirus-1, as shown in this paper, but also for other enteric viruses that have been studied, i.e., rotavirus SA-11, echovirus-12, and coxsackievirus B5 (unpublished data). Finally, growth of microbial contaminants was reduced considerably in cell culture media during plaque assays by the use of additional antibiotics, including gentamicin. The concentrations of antibiotics suggested by this study had no observable effect on the plaquing efficiencies of any of the four enteric viruses that have been tested. These and the other methods developed in this study have been used for investigations of virus survival in fresh waters (Winston and Ward, submitted).

## ACKNOWLEDGMENT

This study was funded in part by the U.S. Environmental Protection Agency under assistance agreement no. R811183.

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